Avirulence Gene Mapping in the Hessian Fly (*Mayetiola destructor*) Reveals a Protein Phosphatase 2C Effector Gene Family

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ABSTRACT

The genetic tractability of the Hessian fly (HF, *Mayetiola destructor*) provides an opportunity to investigate the mechanisms insects use to induce plant gall formation. Here we demonstrate that capacity using the newly sequenced HF genome by identifying the gene (*vH24*) that elicits effector-triggered immunity in wheat (*Triticum* spp.) seedlings carrying HF resistance gene *H24*. *vH24* was mapped within a 230-kb genomic fragment near the telomere of HF chromosome X1. That fragment contains only 21 putative genes. The best candidate *vH24* gene in this region encodes a protein containing a secretion signal and a type-2 serine/threonine protein phosphatase (PP2C) domain. This gene has an *H24*-virulence associated insertion in its promoter that appears to silence transcription of the gene in *H24*-virulent larvae. Candidate *vH24* is a member of a small family of genes that encode secretion signals and PP2C domains. It belongs to the fraction of genes in the HF genome previously predicted to encode effector proteins. Because PP2C proteins are not normally secreted, our results suggest that these are PP2C effectors that HF larvae inject into wheat cells to redirect, or interfere, with wheat signal transduction pathways.

Graphical Abstract

First-instar Hessian fly larvae (A) use their microscopic mouthparts (m) to introduce secreted serine/threonine protein phosphatases (B) into wheat cells. These effectors presumably modulate wheat signal transduction pathways that alter seedling growth and development. (C) Susceptible seedlings (S) are stunted, but seedlings that detect the presence of an effector induce effector-triggered immunity (R) and grow normally.

Key words

Effector protein
Avirulence gene
Plant-gall
Gene-for-gene interaction
Plant resistance
Protein phosphatase

1 Introduction

Plant-galling arthropods, “nature’s most sophisticated herbivores” (Shorthouse et al., 2005), create a protected nutrient-rich environment by modulating plant cell biochemistry and development. The resulting galls are often complex homeotic transformations of plant tissue. Occasionally they are so multifarious that they appear to be novel plant tissues. The precise mechanisms used to induce plant galls are poorly understood. However, evidence is accumulating that suggests that secreted effector proteins are responsible (Giron et al. this issue, Favery et al. this issue)(Aggarwal et al., 2014, Bent and Mackey, 2007, Harris et al., 2015, Hogenhout and Bos, 2011, Hogenhout et al., 2009, Stuart et al., 2012). This suggests that the same mechanisms that defend plants against certain pathogenic effectors issued by plant pathogenic microbes and nematodes are also used to defend plants against arthropod-delivered plant-galling effectors (Favery et al. this issue)(Harris et al., 2015, Hogenhout et al., 2009, Jones and Dangl, 2006, Kaloshian, 2004, Smith and Clement, 2012). Evidence supporting this hypothesis is accumulating (Aggarwal et al., 2014, Kobayashi et al., 2014, Zhao et al., 2015)(Kobayashi, this issue, Bentur et al., this issue).

One line of evidence that supports this hypothesis is the gene-for-gene interaction that has been observed between certain insects and their host plants (Stuart, 2015). This hypothesis has been extensively studied in plant pathology (Bent and Mackey, 2007), and the conceptual mechanisms underlying these relationships are familiar: Loosely analogous to antibody-antigen recognition in a vertebrate host, the products of specific plant resistance (*R*) genes evolve to perceive, either directly or indirectly, specific plant pathogen effectors (Jones and Dangl, 2006). This perception elicits effector-triggered immunity (ETI) in the plant. The genes that encode these “perceivable” effectors are called *Avirulence* (*Avr*) genes because pathogen genotypes that express the effector are unable to colonize plants that carry the corresponding *R* gene. The existence of *Avr* genes in plant parasite populations is evidence that the parasite uses effectors to colonize its host. *Avr* gene mapping was the first method of effector discovery (Staskawicz et al., 1984), and it remains an effective approach (Ellis et al., 2009). Hundreds of effector-encoding genes are believed to reside in the genomes of plant-parasitic insects (Bos et al., 2010, Hogenhout and Bos, 2011, Zhao et al., 2015). A small number of these are *Avr*-encoded effectors.

R proteins may act as sentries that guard the cellular targets of parasite effectors or as decoys against effector activity (Jones and Dangl, 2006, van der Hoorn and Kamoun, 2008). They typically contain nucleotide binding (NB) and leucine rich repeat (LLR) motifs. A few proteins with these structures have been shown to protect plants from insects (Milligan et al., 1998, Rossi et al., 1998)(Kobayashi, this issue, Bentur et al., this issue). Normally the products of different *R* genes perceive effectors that are encoded by different *Avr* genes. Thus, a gene-for-gene relationship is observed in which each plant *R* gene has a different corresponding parasite *Avr* gene. When cognate *R*-*Avr* gene pairs are present in both host and parasite, ETI is elicited, resulting in a resistant, or incompatible, host-parasite interaction. In the absence of any other *R*-*Avr* gene pair, if either the *R* or *Avr* cognate is missing, ETI is not induced. This results in a susceptible, or compatible, interaction. A very small number of plant-insect interactions exist in which such a gene-for-gene relationship has been demonstrated (Stuart, 2015) (Bentur et al. this issue, Kobayashi, this issue). However, it is also true that few plant-insect interactions have the genetic tractability necessary to detect these relationships.

One important plant-insect interaction that does have sufficient genetic tractability occurs between wheat (*Triticum* spp.) and the gall-forming Hessian fly (HF, *Mayetiola destructor*). The HF is an important pest of a major commodity; so over 34 different HF *R* genes have been

genetically identified (Hao et al., 2013, Li et al., 2013, Li et al., 2015, McDonald et al., 2014). Methods to clone these genes in wheat are advancing (International Wheat Genome Sequencing, 2014). HF *R* genes all have the designation “*H*” (for HF) and then each is given a unique name or number, e.g. *H13*, *H24* and *Hdic*. Genetic analyses performed on the HF suggest that for each *H* gene examined, a different cognate *Avr* gene exists in the insect (Stuart et al., 2012). These *Avr* genes are named by placing a “*v*” (for “virulence to”) in front of the name of its cognate *R* gene, e.g. *vH13*, *vH24* and *vHdic*, because recessive mutations in these *Avr* genes defeat resistance (Harris et al., 2003). Compatible and incompatible wheat-HF interactions can be observed on wheat seedlings, and individual seedlings can support multiple (>20) larvae. This makes it possible to establish and evaluate hundreds of wheat-HF interactions in a relatively small space. The gene-for-gene nature of the interaction was first observed using classical genetics. The ability to identify and clone a HF *Avr* gene was accomplished decades later, after the development of HF bacterial artificial chromosome (BAC) libraries and BAC-end sequencing (Aggarwal et al., 2014). The map-based approach used in that investigation has subsequently evolved with further advances in genetic technologies (Zhao et al., 2015). The approach also benefits from the insect’s polytene chromosomes and its relatively small genome (Stuart et al., 2012). The HF reference sequence and associated genome browser (https://i5k.nal.usda.gov/Mayetiola_destructor) facilitate this further, by making it possible to both easily develop molecular markers and identify genes in any region of the HF genome.

Although the HF-induced gall is not the characteristic outgrowth or swelling that is associated with many common galls, it is very much a gall in the sense that the physiology of the plant is altered and a nutrient tissue is created (Harris et al., 2010, Harris et al., 2006, Harris et al., 2003). The plant acts as the source of photosynthates and other nutrients and the developing HF larvae act as the sink that receives those substances (Harris et al., 2015, Stuart et al., 2012). In only 4 days, a single larva (<1 mm in length), secretes powerful substances that permanently cause the entire seedling to cease cell elongation, stop cell division, dramatically alter plant metabolism and severely increase cell permeability; all for the benefit of the larva (Liu et al., 2007, Stuart et al., 2012, Williams et al., 2011, Zhu et al., 2008). Increasing the expression of at least one wheat gene, *Md susceptibility-1* (*Mds-1*), is critical to larval survival and plant stunting (Liu et al., 2013). We suspect, therefore, that altering the expression of *Mds-1* and other “susceptibility genes” may be the mechanism HF effectors use to modulate plant developmental pathways and that this is key to the power of the larval secretions.

To explore the hypothesis that insect effectors are used to induce plant-gall formation, candidate effectors have been identified in the larval salivary gland transcriptome (Chen et al., 2010) and the HF genome has been sequenced (Zhao et al., 2015). We have also exploited the genetic tractability of the HF to genetically map effector-encoding *Avr* genes (Aggarwal et al., 2014, Zhao et al., 2015). Our intent here is to describe the facility of map-based *Avr* gene discovery in the HF as a means of identifying insect proteins that are capable of reprogramming plant development and immunity. These methodologies will be reviewed as a novel candidate HF *Avr* gene, *vH24*, is mapped and described. *vH24* encodes the effector that elicits ETI in wheat seedlings carrying *R* gene *H24*. It is presumed that this effector benefits the insect as a virulence factor in the absence of *H24*. *H24* itself was identified in the diploid wheat *T. tauschii*, and transferred into bread wheat (*T. aestivum*) via a wide cross (Raupp et al., 1993). It was mapped with respect to molecular markers on the long arm of wheat chromosome 3D (Ma et al., 1993). Although it has not been deployed in wheat cultivars in the United States, surveys of U.S. HF populations indicate that *H24*-virulence is not uncommon (Chen et al., 2009).

The ability to perform map-based *Avr* gene identification in the HF puts insect plant parasites on par with microbial plant pathogens, whose smaller genomes and rapid reproduction have permitted *Avr* gene cloning for decades (Staskawicz et al., 1984). Although these are not the only methods that can be applied to identify proteins and other molecules that plant parasites use to modulate plant development and immunity, they remain an effective and tested means of effector identification. Modern sequencing technologies are rapidly adding sequenced insect genomes to the arsenal of tools we have to investigate plant-insect interactions (Richards and Murali, 2015). By combining these technologies with other plant-parasitic insects that are amenable to genetics, we can expect the approach to become more effective in the identification of genetic traits that underlie the phenotypic differences that are observed among many plant-insect interactions. Our understanding of gene-for-gene and other plant-insect interactions will certainly benefit, as the contributions of Kobayashi () and Bentur et al. () in this issue clearly attest.

2 Materials and Methods

2.1 *H24-virulent, H24-avirulent and structured HF mapping populations*

All HFs were reared as the offspring of individual females on separate caged pots of wheat seedlings as previously described (Rider et al., 2002). All HF strains, matings and families were reared at 18 °C under a 12-h light: 12-h dark photoperiod. *H24-virulent* and *H24-avirulent* strains were selected from an Israeli HF strain maintained in the USDA HF research program at Purdue University.

To select the *H24-avirulent* strain, single mated females from the Israeli HF population were allowed to deposit their eggs on leaves of wheat seedlings growing in 36 caged pots. Each pot contained HF-susceptible 'Newton' seedlings growing on one side of the pot and the *H24-resistant* seedlings in the other side of the pot. The seedlings and the larvae in each pot were examined 10 days after egg deposition for plant stunting and larval survival. Pots that had either stunted *H24*-seedlings or surviving larvae on *H24*-seedlings were discarded. Larvae on the Newton seedlings on the remaining pots were allowed to develop, emerge as adults and intermate. Individual mated females were then caged on separate pots of Newton and *H24*-seedlings for a second generation of selection for *H24-avirulence*. This procedure was followed for three generations, after which there was no evidence of *H24-virulence* in the population. This population was subsequently maintained as the "*H24-avirulent strain*" reared as offspring of single females on caged pots of Newton seedlings.

To select the *H24-virulent* strain, we followed standard procedures (Zantoko and Shukle, 1997). Briefly, mated HFs collected from the Israeli population deposited their eggs on *H24-resistant* seedlings. Surviving larvae on stunted seedlings were allowed to develop into adults. These adults were allowed to intermate and the mated females deposited their eggs on *H24-resistant* seedlings in caged pots. This process was repeated for a third generation in order to increase the frequency of *H24-virulence*. The first-instar larval offspring of the surviving females were then collected individually in 10 µL drops of water containing 0.02% NP-40 and placed, one larva per plant, on the youngest leaf of single *H24*-seedlings. Using a single larva per plant insures that avirulent larvae, which can survive on the nutritive tissue created by a virulent larva on the same plant (Harris et al., 2006, Williams et al., 2011), are eliminated from the population. Larvae that survived alone on the *H24* seedlings were used to establish the *H24-virulent strain*. This strain was maintained on *H24-resistant* plants under the conditions described above.

Three independent structured mapping populations (F2A, F2B and F4C) were developed from crosses between virulent and avirulent males and females as previously described (Aggarwal et

al., 2014). HF females typically produce offspring of only one sex (Benatti et al., 2010). Therefore each mapping population was initiated by crossing two P₁ sister *H24*-virulent (*v/v*) females to the same P₁ *H24*-avirulent (*A/-*) male (Figure 1A). One P₁ female produced F₁ daughters and the other produced F₁ sons. After mating and egg deposition, the P₁ male and females were collected and the genomic DNA of each P₁ HF was extracted separately. The F₁, and all subsequent generations, were reared on Newton seedlings and allowed to freely intermate. To map *H24*-virulence relative to molecular genetic markers, males were collected from the F₂ and F₄ generations and individually genotyped as *H24*-virulent or *H24*-avirulent in separate testcrosses (Figure 1B). Males were collected immediately after mating and placed at - 80 °C in separate 0.5 mL plastic tubes for future genomic DNA extraction.

Fig. 1. HF males taken from structured mapping populations are genotyped as virulent or avirulent to HF *R* gene *H24* in wheat. (A) Mapping populations are initiated with matings between homozygous virulent (*v/v*) sister females and a single avirulent (*A/-*) male. Their offspring are allowed to freely intermate. Males are collected for genotyping after the F₁ generation (horizontal arrows). (B) Collected males are individually genotyped in testcrosses with homozygous virulent females (*v/v*). *H24*-avirulent (*A/-*) males produce female offspring that are incapable of stunting *H24*-plants (R). *H24*-virulent (*v/-*) males produce female offspring that are capable of stunting *H24*-resistant plants (S). Newton (N) wheat seedlings are used as a susceptible check.

Testcrosses consisted of individual males mated to a single homozygous *H24*-virulent females, collected from the *H24*-virulent strain, caged together on separate pots containing *H24* and Newton seedlings growing in separate halves of the pots (Figure 1B). The Newton seedlings served as a check for infestation. Because each female produced offspring of only one sex, the adults that emerged from the Newton seedlings allowed us to determine the sex of each testcross family. If the testcross offspring were female and unable to live on *H24* plants, the male was scored as “*H24*-avirulent.” If the testcross offspring were female and able to live on *H24* plants, the male was scored as “*H24*-virulent.” If the testcross offspring were males, the genotype of the male could not be determined.

2.2 Molecular genetic mapping

Molecular markers were developed and genetic and physical mapping were performed as described previously (Aggarwal et al., 2009, Aggarwal et al., 2014). Briefly, primers flanking simple sequence repeats (SSRs) in genomic sequence were designed and synthesized to produce short amplicons (100 to 400 bp). These were used to PCR-amplify genomic DNA isolated from males genotyped as *H24*-virulent and *H24*-avirulent in separate reactions. We found that 28% of the primer pairs flanking SSRs on the X chromosomes produced amplicons with polymorphic lengths visible on 2.5% agarose gels (Table S1). Primers used to position *H24*-virulence within scaffold X1R.8 are presented in Table S2. The amplicons produced using

P₁ male and female genomic DNA determined the phase of the polymorphisms. HF bacterial artificial chromosomes (BACs) containing the polymorphic sequences were used as probes against the HF polytene chromosomes in fluorescence *in situ* hybridization (FISH) experiments in order to confirm the genomic locations of the markers. Sequenced BAC-ends allowed us to determine the location of the BACs in HF sequenced scaffolds.

2.3 Analysis of candidate *vH24* gene transcription

Total RNA was isolated from two-day-old first-instar larvae of the *H24*-avirulent strain using the RNeasy Mini Kit (Qiagen). The sequences of the oligonucleotide primers used in RT-PCR experiments are presented in Table S3. To synthesize the first-strand cDNA, 0.5 µg of total RNA was mixed with 10 pmol of SMART IV oligo DNA and 10 pmol of (dT)17-adaptor oligo DNA (Table S3) in a total volume of 5 µl. The mixture was heated to 72°C for 2 min. and then chilled on ice for 2 min. To synthesize the full-length first-strand cDNA, we used the SMARTScribe kit (Clontech) according to the manufacturer's instructions. Briefly, 5 µL of first-strand buffer, 1 µL of 20 mM DTT, 1 µL of dNTP mix (10mM), and 1 µL of SMARTScribe MMLV reverse transcriptase (Clontech) were added to the tube to bring the total volume to 10 µL. The mixtures were incubated at 42°C for 1 h using the SMART IV oligo to amplify from the 5'-end and the (dT)17-adaptor to amplify from the 3'-end of the single stranded transcripts. To generate full-length double-stranded cDNA, long-distance PCR was performed using the Advantage 2 polymerase Mixes (Clontech), as recommended by the manufacture, and forward primer 5'-RACE-F and reverse primer (dT)17-adaptor. PCR conditions were 95 °C for 1 min followed by 22 cycles of 95 °C for 15 s and 68 °C for 6 min.

Rapid Amplification of cDNA Ends (RACE) was performed in both the 5' and 3' directions as nested PCR experiments. The initial 5'-RACE was performed using the forward primer 5'-RACE-F, the reverse primer Mdes001440-5RACE-R1 and synthesized double-stranded cDNA. The nested PCR used primers 5'-RACE-F and Mdes001440-5RACE-R2 and the product from the initial 5' reaction as template. To perform 3'-RACE, the first reaction used primers Mdes001440-3RACE-F1 and 3'-adaptor-R. The nested reaction used primers Mdes001440-F and 3'-adaptor-R and product from the initial 3'-reaction as template. The amplicons produced were cloned into plasmid vector pCR4-TOPO (Invitrogen) and sequenced at the Purdue Genomics Center.

2.4 PCR-walking

To clone and sequence genomic DNA upstream of the virulence-associated allele of candidate gene *Mdes001440*, we used TOPO-vector-ligation PCR (TVL-PCR) to perform a short sequence walk (Orcheski and Davis, 2010). To prepare template for TVL-PCR, 0.6 µg of *H24*-virulent genomic DNA was digested to completion with *EcoRI* (New England Biolabs) and then end-repaired using GoTaq DNA polymerase (Promega) according to the manufacturer's instructions. This created 3'-adenine-overhangs on each DNA fragment. The fragments were cleaned using the QIAquick PCR purification kit (Qiagen) and ligated into the pCR4-TOPO vector (Invitrogen). Nested PCR was then performed using this template. Two separate first-round reactions were performed, using oligonucleotides complementary to the M13F and M13R sites in separate reactions with the candidate *Mdes001440*-specific oligonucleotide *Mdes001440*-R. The products of these reactions were used as template in two separate PCR experiments. The first used an oligonucleotide complementary to the T7 site and primer *Mdes001440*-R1. The second used an oligonucleotide complementary to the T3 primer site and *Mdes001440*-R1. The amplicons generated in these experiments were cloned into plasmid vector pCR4-TOPO and sequenced at the Purdue Genomics Center.

3 Results

3.1 An Israeli HF population permits the isolation of H24-virulent and H24-avirulent strains

Over several decades, HF strains, races or “biotypes” have been selected from natural populations that differ with respect to their ability to survive on and stunt wheat seedlings with different genes for resistance (Gallun et al., 1961). These have been used to both identify new *R* genes and to determine the effectiveness of *R* genes bred into elite cultivars. Virulence to *H24* was extremely low to non-existent in the Purdue University greenhouse populations. We therefore sought *H24*-virulence in a population collected in Israel and maintained in the USDA-ARS greenhouse at Purdue University. We reasoned that chromosome rearrangements might interfere with recombination analyses involving crosses between U.S. and Israeli HF populations. To avoid this possibility, a complementary *H24*-avirulent population (AvrH24) was isolated from the same Israeli HF population.

Approximately 1000 *H24*-seedlings were infested with 150 females arbitrarily selected from the Israeli population. The females deposited all of their eggs (>2000) on the *H24*-seedlings and the eggs were allowed to eclose and the neonate larvae were allowed to attempt to establish nutritive tissue at the base of the plants. Among all of the infested plants, 15 had a susceptible reaction. Assuming the susceptibility of each of these plants was due to a single *H24*-virulent larva, we estimated that less than 1% of the Israeli population is *H24*-virulent. Larvae surviving on the susceptible seedlings were allowed to develop into adults and used to create an *H24*-virulent strain (virH24) as described in the Materials and Methods. We detected no phenotypic differences in compatible interactions between *H24*-virulent and *H24*-avirulent larvae on Newton seedlings.

3.2 *H24*-virulence is a recessive X-linked trait

Understanding the unusual inheritance pattern in the HF is essential to mapping virulence mutations (Fig. 2). By convention, the chromosomes composing a HF zygote’s genome are grouped into eight “S” and a variable number (28-32) of “E” chromosomes (Stuart et al., 2012). The S chromosomes are divided further into two autosome pairs (A1 and A2) and two “X” chromosome pairs (X1 and X2). S chromosomes undergo meiotic recombination in females, are sexually inherited and are present in both the soma and the germ line. The E chromosomes are maternally inherited and germ-line-limited. Because they have no impact on the inheritance of virulence, they are given little attention here.

Fig. 2. Chromosome elimination and sex determination in the Hessian fly. S chromosomes (A1, A2, X1 and X2) are shown as black (maternally inherited) and grey (paternally inherited) vertical lines. E chromosomes are shown as vertical hatched lines.

Zygotes receive one copy of each S chromosome from their father (A1A2X1X2). Because males transmit only their mother's S chromosomes, all of the offspring of a single male are isogenic with respect to these chromosomes. Zygotes receive one copy of each S chromosome and a complement of E chromosomes from their mother (A1A2X1X2E, where E represents 28-32 E chromosomes). Because S-chromosome recombination occurs during oogenesis, zygotes differ with respect to alleles on these chromosomes. Chromosome elimination during embryogenesis determines the sex of an individual; individuals that retain a diploid complement of the S chromosomes (A1A2X1X2/A1A2X1X2) develop as females, but individuals that eliminate the paternally derived X chromosomes (A1A2X1X2/A1A2OO) develop as males. Maternal genotype determines whether the paternal X chromosomes are retained or eliminated (Benatti et al., 2010). Females carrying a small autosomal inversion (W') cause paternal X-chromosome retention (Fig. 2). As a consequence, all of the offspring of W' -bearing females develop as females. Females lacking W' condition their offspring to eliminate the paternally inherited X chromosomes, and as a result, all of their offspring develop as males.

Utilizing this knowledge, virulence can be readily assigned to autosomes or X chromosomes as a recessive trait (Fig. 3). If matings between virulent females and avirulent males produce avirulent male offspring, virulence is autosomal. However, if those matings produce virulent male offspring, virulence is X linked.

Fig. 3. All possible male and female genotypes resulting from crosses between homozygous virulent (v/v) females and avirulent ($A/-$) males are illustrated. When the virulence mutation is X linked, male offspring are able to survive on resistant plants because they lack the dominant avirulent allele (A).

We made 13 independent crosses between AvrH24 males and virH24 females. Each mated female was allowed to oviposit on a caged pot containing *H24* and 'Newton' (fully susceptible) seedlings on opposite sides of the pot. Newton seedlings were provided to assure that some F_1 offspring in each F_1 family survived to the adult stage so that the sex of each family could be easily determined. A family was considered *H24*-virulent if the larvae survived on and stunted *H24* seedlings. A family was considered *H24*-avirulent if the larvae died and failed to stunt *H24* seedlings. We observed only two types of F_1 families: seven were *H24*-avirulent and female, and six were *H24*-virulent and male. These data indicate that *H24*-virulence is an X-linked

internal controls in each reaction. (E) PCR amplification of *Mdes001440* and *Mdes001447* using DNA extracted from *H24*-virulent and -avirulent individuals. Note that the virulence-associated DNA fails to amplify well with primers designed to amplify *Mdes001440*, but there is no such association with *Mdes001447*.

To test this possibility, we considered each of six scaffolds that had previously been physically positioned in the “W” segment of chromosome X1 (Fig. 4C), but had not been ordered with respect to other scaffolds on the chromosome (Aggarwal et al., 2009). Scaffold X1Random.8, the longest of the six, was examined first. Fluorescence *in situ* hybridization of X1Random.8 BAC clones indicated the scaffold was very near the X1 telomere (Fig. 4C). Using the F2A population, the genetic positions of thirteen X1Random.8 markers (Table S2) were determined. These data confirmed that X1Random.8 is distal to scaffold X1.7 (Fig. 4B). In addition, nine markers within a 620-kb X1Random.8 segment failed to recombine with *H24*-virulence, suggesting that mutations conditioning *H24*-virulence reside between markers X1R.8-81 and X1R.8-148 on the X1Random.8 scaffold.

To test this possibility further, we developed two additional mapping populations, F2B and F4C. The F2B population consisted of 39 F₂ males and the F4C population consisted of 66 F₄ males. Each male was genotyped with respect to *H24*-virulence and polymorphisms at scaffold X1.7 and X1Random.8 markers (Fig. 4B). These populations resolved the position of *H24*-virulence to a 230-kb sequence between markers X1R.8-81 (base 1,121,087) and X1R.8-99 (base 1,351,496), within the 620-kb sequence identified using population F2A (Table S2). This result was consistent with the hypothesis that loss-of-function mutations in a single effector-encoding *Avr* gene defeat *H24*-resistance.

3.4 Genic analyses reveal a single *vH24* candidate

To test this hypothesis further, we identified the genes between markers X1R.8-81 and X1R.8-99 and examined them for a signal peptide, a feature that is commonly associated with eukaryotic effectors. Importantly, the interplay *R* and *Avr* genes during the coevolution of host and parasite can result in diversifying selection for alleles at both loci (Allen et al., 2004). We therefore used evidence of diversifying selection, an absence of sequence similarities to other proteins, to help us identify candidate HF *Avr* genes.

Using Web Apollo (Lee et al., 2013) and the HF sequence data maintained at the i5k workspace (https://i5k.nal.usda.gov/Mayetiola_destructor), 21 putative genes were discovered and manually annotated within the *H24*-virulence-associated 230-kb segment (Table 1). The signal peptide prediction algorithm, SignalP4.1 (Petersen et al., 2011), indicated that only four of these genes encode signal peptides. BLASTP (Altschul et al., 1990) sequence alignments indicated that only one of these genes (*Mdes001440*) has the low sequence conservation expected of a rapidly evolving effector-encoding *Avr* gene. *Mdes001440* is composed of a single exon, and this encodes a protein that has less than 27% amino acid identity with its best hit, a vertebrate type 2C protein phosphatase (PP2C; also known as metal ion-dependent protein phosphatases, PPM). It lacks significant amino acid sequence similarities to all arthropod genes in the NCBI non-redundant protein database.

Expression analysis supported the hypothesis that *Mdes001440* is *vH24*. Using total RNA separately extracted from *H24*-virulent and *H24*-avirulent larvae, reverse-transcription PCR, (RT-PCR) was performed using primers designed to specifically amplify transcripts of *Mdes001440* (primers: *Mdes001440*-F and *Mdes001440*-R; Table S3) and *Mdes001447* (primers: *Mdes001447*-F and *Mdes001447*-R). Consistent with predicted *Avr* gene loss-of-function, there was no evidence of *Mdes001440* transcription in *H24*-virulent first-instar larvae, although transcript amplification was evident in *H24*-avirulent first-instar larvae (Fig. 4D).

Mdes001447 expression was evident in both *H24*-virulent and *H24*-avirulent larvae, as was the expression of the internal control (HF ubiquitin) in all reactions.

Table 1

Genes residing within the 230-kb *H24*-virulence-associated region of the HF genome.

Mdes no. ¹	Position ²	Length ³	SP ⁴	BLASTp hit ⁵	Locus	E-value
001435	+1167578	1323	+	Ccap; dystroglycan-like protein DG	XP_004535783	0.0
001436	+1183611	743	-	Dgrim; GJ20080	XP_001987700	6e-103
001437	-1185696	174	-	Mdom; ATP synthase subunit d	XP_005175277	4e-62
001438	+1189873	174	-	Amel; ras-like GTP-binding protein	XP_006570782	7e-76
001439	+1183271	440	-	None	None	None
001440	-1203662	266	+	Trub; serine/threonine phosphatase	XM_003975587.1	0.004
001441	+1206881	891	-	Ccap; transportin-1-like	XP_004526941	0.0
001443	-1219619	239	-	Cquin; transport protein Use1	XP_001841767	4e-81
001444	+1219983	597	-	Dmel; separase	NP_523935	1e-40
001445	-1228195	1272	-	Cflor; TE P transposase	EFN62483	5e-22
001446	+1229135	214	-	Dmel; Rab19-like protein	NP_523970	9e-85
001447	+1237602	369	+	Dmel; CG7201, isoform A	NP_648200	7e-100
001448	+1276852	881	-	Aaeg; neurotactin	XP_001659857	0.0
001449	-1293243	1930	+	Dmel; plexin A, isoform A	NP_524637	0.0
001450	-1304015	345	-	Dmel; UDP-sugar transporter	BAB62105	3e-156
001451	+1298401	302	-	Cquin; NTKL-binding protein 1	XP_001870841	6e-45
001452	-1301589	261	-	Aaeg; ubiquinone biosynthesis protein	XP_001649247	1e-116
001453	-1308530	369	-	Aaeg; spermine synthase	XP_001653177	9e-102
001454	+1311513	725	-	Aaeg; type iv inositol 5-phosphatase	XP_001653179	0.0
001455	+1317832	874	-	Mdom; palmitoyltransferase-like	NP_001137938	0.0
001457	+1329733	411	-	Cquin; lim homeobox protein	XP_001843929	1e-124

¹After manual annotation of the Mdes 1.0 official gene set.

²Base corresponding to translation start site in scaffold X2Random.8; positive (+) and negative (-) strands.

³Number of amino acid residues.

⁴Signal peptide present (+) or absent (-)

⁵Aaeg = *Aedes aegypti*, Amel = *Apis mellifera*; Ccap = *Ceratitis capitata*, Cflor = *Camponotus floridanus*, Cquin = *Culex quinquefasciatus*, Dgrim = *Drosophila. grimshawi*; Dmel = *D melanogaster*, Mdom = *Musca domestica*; Trub = *Takifugu rubripes* (tiger puffer fish)

We also performed 5'- and 3'- RACE to obtain the complete sequence of *Mde001440* transcripts. The result was in agreement with our previous genomic annotation of the gene (Fig. S1). The *Mdes001440* transcript is 1026 bp long and contains an 801-bp coding region. Its predicted protein is composed of 266 amino acid residues, which includes a putative secretory signal of 25 amino acids at the N-terminus (Fig. S1). Although *Mdes001440* has little sequence similarity to genes in other organisms, structural alignments indicated that it encodes a protein with an imperfect PP2C Serine (Ser)/Threonine (Thr) protein phosphatase domain (Fig. 5). In this alignment, only one of the eight residues present at the active site failed to map to the residues that compose the canonical PP2C domain.

Oligonucleotide primers used to analyze *Mdes001440* and *Mdes001447* gene expression were used to amplify genomic copies of the genes from both *H24*-virulent and *H24*-avirulent individuals. These experiments indicated that allelic sequence variation exists between *H24*-virulent and *H24*-avirulent individuals at the *Mdes001440* locus (Fig. 4E). To discover the source of this variation, we cloned *Mde001440* from DNA isolated from *H24*-virulent individuals using a combination of PCR and TVL-PCR sequence walking. A 163-bp DNA insertion, 134-bp upstream of the transcription start site (Fig. S1), was the only difference observed between virulent- and avirulent-associated sequences. This insertion is flanked by an 8-bp short direct repeat followed by a 4-bp inverted repeat and is present in seven different scaffolds of the HF reference sequence (BLASTN $\leq 4e-81$), suggesting that it is the remnant of a class II

transposable element. The insertion presumably changes the tertiary conformation of the gene, which interferes with both its transcription (Fig. 4D) and PCR amplification (Fig. 4E).

Fig. 5. Predicted structure of the protein encoded by candidate *vH24* gene *Mdes001440*. (A) Phyre2 (Kelley and Sternberg, 2009) predicted structure of 191 out of 266 amino acids modeled with 99% confidence. (B) Structural alignment of *Mdes001440* using RSP-BLAST (Altschul et al., 1997) and human serine/threonine phosphatase 2C (K.Das et al., 1996). Blue residues are structurally aligned. Red residues (near the active site) are identical and structurally aligned. Grey residues are unaligned. Grey spheres represent manganese or magnesium ions.

3.5 Candidate *vH24* is a member of a small effector-encoding gene family

Searching the HF reference sequence genome revealed 17 other genes and gene fragments that have significant sequence similarity to *Mdes001440* (TBLASTN $\leq 1.0e-8$). The sequence similarities are associated within both the PP2C motif and the secretion signal (Fig. S2). A secretion signal is lacking in three of these genes. However, gaps in the reference HF genome sequence truncate each of these genes at their 5'-ends, so it is likely that the signal-peptide-encoding sequences of these genes are simply masked by these gaps. Two of the 17 genes identified are composed of two exons, but like *Mdes001440*, at least 12 others are composed of a single exon. Ten of the genes are located on chromosome A2; five of these exist in tandem on scaffold A2.12, three are clustered on scaffold A2.1 and two are located in tandem on scaffold A2.8. All 18 genes exist within the “self-homology” genic fraction of the HF genome that was previously predicted to encode effectors (Zhao et al., 2015). Thus, our map-based approach to *vH24* discovery appears to have uncovered a small family of putative PP2C-like effector encoding genes.

To examine the origin of this gene family, we used TBLASTN to identify putative homologs in the *Drosophila melanogaster* (*Drosophila*) genome. Interestingly, whereas two of the HF genes had no apparent homologs in the *D. melanogaster* genome, 16 others were similar in sequence to *Drosophila* gene CG7115 (Table 2). These similarities were present only within the PP2C domain. CG7115 lacks a secretion signal. We also used TBLASTN to search the HF genome for homologs to each of the 15 PP2C encoding *Drosophila* genes (Table 2). These genes include *fos intronic gene* (*fig*, CG6036), *Ppm1* (CG12169), *PH domain leucine rich repeat protein phosphatase* (CG10493), *Pyruvate dehydrogenase phosphatase* (CG12151), and *alphabet* (*alph*, CG1906). The best hits to 14 of these *Drosophila* genes, including CG7115, do not belong to the putative PP2C-effector family (Table 2). The best hit of the *Drosophila* gene *alph* was putative HF PP2C-effector (*Mde008053*). However, *alph* is a small gene whose true

HF ortholog may be *Mdes004102* (TBLASTN = 6e-23), which is also outside of the putative HF PP2C-effector family. Taken together, these observations lead us to conclude that the putative HF PP2C-effector family arose from an expansion of an insect PP2C domain after it became aligned with a signal peptide encoding exon. Evolution of the genes in the PP2C-effector family has made *Mdes001440* and two other HF genes less recognizable as insect PP2C homologs.

Table 2

TBLASTN “best hits” (BH) of PP2C encoding genes in HF and *D. melanogaster* (Dm) genomes.

TBLASTN to Dm annotated genes				TBLASTN to HF genome sequence			
HF query	Dm BH	E-value	SP ¹	Dm query	HF BH	E-value	SP ¹
Mdes001440	CG7115	0.052	No	CG7115	Mdes008796	1e-54	No
Mdes007548	CG7115	1e-26	No	CG17598	Mdes000375	1e-176	No
Mdes003813	None		No	CG10376	Mdes011060	2e-31	No
Mdes017452	CG7115	3e-04	No	CG12169	Mdes006632	1e-41	No
Mdes017453	CG7115	5e-07	No	CG17746	Mdes006632	7e-42	No
Mdes008052	CG7115	5e-50	No	CG10493	Mdes000801	9e-14	No
Mdes008053	CG7115	2e-44	No	CG2984	Mdes000336	1e-70	No
Mdes001891	CG7115	1e-45	No	CG15035	Mdes006807	6e-47	No
Mdes010556	CG7115	3e-46	No	CG9801	Mdes007315	1e-153	No
Mdes017437	CG7115	6e-39	No	CG6036	Mdes004102	2e-22	No
Mdes001889	CG7115	3e-31	No	CG7615	Mdes006807	8e-26	No
Mdes001892	CG7115	2e-39	No	CG1906	Mdes008053	2e-24	Yes
Mdes001893	CG7115	1e-49	No	CG1215	Mdes004387	1e-118	No
Mdes012615	CG7115	3e-40	No	CG10417	Mdes004102	6e-66	No
Mdes014325	CG7115	2e-22	No	CG12091	Mdes006807	6e-45	No
Mdes010558	CG7115	1e-44	No				
Mdes017386	None		No				
Mdes015322	CG7115	1e-36	No				

¹Absence (No) or presence (Yes) of a secretion signal in the BH sequence (SignalP 4.1).

4 Discussion

Although functional assays to test *Mdes001440* for effector activity have yet to be performed, we are confident that *Mdes001440* is *Avr* gene *vH24* given the following observations: 1) *Mdes001440* was mapped within a genomic fragment of only 230 kb that contains only 21 genes. 2) It was one of only four genes within that fragment encoding a secretion signal. 3) It was the only gene within that fragment displaying the type of sequence diversity expected of an effector-encoding gene. 4) It belongs to the genic fraction of the HF genome predicted to contain effector-encoding genes and the only gene in the mapped fragment that belongs to this group. 5) It is expressed in *H24*-avirulent first instar larvae, but is not expressed in *H24*-virulent first instars. 6) An insertion near the gene’s promoter segregates perfectly with *H24*-virulence and corresponds to a loss of the gene’s expression in virulent individuals.

Finally, the secretion of PP2C protein phosphatases into plant cells is congruent with effectors that redirect, or interfere, with a plant cell’s ability to respond to environmental or chemical cues. PP2C proteins reverse the protein phosphorylation activities of protein kinases in stress-related signal transduction pathways (Moorhead et al., 2009, Shi, 2009). They are largely conserved among animals and appear to have been acquired by some bacteria via lateral gene transfer (Zhang and Shi, 2004). The human genome contains 18 PP2C genes with orthologs in other vertebrates (Kerk et al., 2008, Shi, 2009). Plants have a much greater abundance of PP2C encoding genes, with 80 in *Arabidopsis*, 90 in rice (*Oryza sativa*) and 130 in corn (*Zea mays*) (Fuchs et al., 2013, Yang et al., 2010). Few, if any, native PP2C proteins have a signal peptide, which suggests that the candidate *vH24* protein’s secretion signal is there to permit it to function as an effector in plant cells.

Some plant PP2Cs act as co-receptors of the plant hormone abscisic acid (ABA). This suggests a potential role for the *vH24*-encoded protein as a modulator of plant development. ABA plays regulatory roles in a number of plant physiological processes. It regulates plant responses to stress and has been implicated in the mediation of responses to plant wounding and disease (Fuchs et al., 2013, Schweighofer et al., 2007, Spartz et al., 2014, Sugimoto et al., 2014). ABA binding deactivates the phosphatase activity of PP2C co-receptors, which ultimately derepresses ABA response genes (Hirayama and Umezawa, 2010). Therefore, the role of the *vH24*-encoded protein may be to interfere, disrupt, or alter one or more ABA-associated pathways to the benefit of the larva. Precedence for a PP2C effector has been observed in infectious *Streptococcus pyogenes* bacteria that use a PP2C protein as a virulence factor in human disease (Agarwal et al., 2012).

vH24 is the fourth candidate *Avr* gene identified in the HF. The ability of *vH13*, the first arthropod *Avr* gene discovered, to elicit plant resistance was demonstrated using RNAi gene silencing (Aggarwal et al., 2014). Attempts to silence candidate *Avr* genes *vH6*, *vH9* (Zhao et al., 2015) and *vH24* using the same methodology were unsuccessful. Nevertheless, in perfect agreement with the gene-for-gene hypothesis, loss-of-function mutations in all four *Avr* genes defeat plant resistance to a different HF *R* gene in wheat. Like *vH13*, (Aggarwal et al., 2014), *vH24* was not previously observed in the transcriptome of first-instar HF salivary glands. Therefore, although examining the transcriptome of the larval salivary gland has uncovered hundreds of putative effectors (Chen et al., 2010), it has not yet revealed the full effector complement the insect uses to attack its host.

The HF PP2C-effector family to which *vH24* belongs is the second HF gene family associated with *Avr* gene mutations. The other gene family, SSGP-71, encodes plant ubiquitin-E3-ligase mimics containing F-box and leucine rich repeat (LRR) domains (Zhao et al., 2015). We suspect that these gene families exist to provide both structural diversity and functional redundancy. Structural diversity allows a low frequency of virulence-associated loss-of-function mutations to preexist in a HF population, but the loss of a single effector does not compromise the ability of an individual insect to survive. Functional redundancy allows for the loss of an effector that is recognizable within a plant genotype that expresses the cognate *R* gene. Other effectors that are sufficiently different in structure go unrecognized even though they have the same or a similar function in the plant.

5 Conclusions

The ecology of host-parasite interactions puts two fundamental physiological processes in conflict: immunological mechanisms that protect the host from foreign organisms and virulence mechanisms that allow parasites to survive on their hosts. Insect-induced plant galls are fascinating manifestations of this conflict. The utility of a completely sequenced HF genome combined with the ability to perform map-based gene discovery in the insect makes the HF a potent experimental organism for plant-insect interactions and plant-gall formation. We have illustrated here how these methodologies permit the discovery of insect effectors that elicit ETI. Genetic mapping and gene expression data identified an insertion in a HF single gene (*vH24*) that is perfectly associated with the ability of HFs to survive on wheat seedlings carrying the *R* gene *H24*. *vH24* expression is also perfectly congruent with *H24*-virulent and *H24*-avirulent genotypes. *vH24* is a member of a small family of genes that appear to encode secreted type 2 Ser/Thr protein phosphatases. This suggests the insect uses secreted PP2Cs as effectors that modulate plant defenses and development by redirecting or interfering with signal transduction

pathways. Definitive proof that *vH24* is the *H24* cognate *Avr* gene awaits the development of a functional assay.

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